

STUDIES ON THE MODE OF ACTION OF AGROCIN 84

Sir:

Agrocin 84, a bacteriocin produced by *Agrobacterium radiobacter* was found to possess lethal action against some oncogenic strains of *A. tumefaciens*. STONIER¹⁾ reported on the presence of this antibiotic-like substance and it was first isolated from solid media in a partially purified state by HEIP, CHATTERJEE, VANDEKERCKHOVE, MONTAGU and SCHELL²⁾. Recently,

the bacteriocin was purified by ROBERTS *et al.*³⁾ and the structure has been established as a 6-N-phosphoramidate of an adenine nucleotide analogue. During the recent years studies have been carried out extensively on the mode of action of other bacteriocins like colicin, megacin, pyocin *etc.*⁴⁾, but the mode of action of any bacteriocin of phytopathogenic bacteria is not so far studied in detail. In this communication an attempt has been made to study the effect of agrocin in agrocin-sensitive [*A. tumefaciens* S1005 (TIP Kerr 14)] and also

Fig. 1. Effect of agrocin on the biosynthesis of macromolecules

Agrocin sensitive *A. tumefaciens* S1005 (TIP Kerr 14) and agrocin resistant *A. tumefaciens* S1005 (TIP Kerr 14) R were grown in the medium A'KO.3 of DUBIN *et al.*⁹⁾ with slight modification. The medium was supplemented with adenine (40 $\mu\text{g/ml}$), guanine (30 $\mu\text{g/ml}$), cytosine (30 $\mu\text{g/ml}$), uracil (10 $\mu\text{g/ml}$) and thymine (10 $\mu\text{g/ml}$). After the third subculture, the cells at early log phase were washed with 0.1 M phosphate buffer, pH 7.8 and resuspended in 'phosphate buffer medium' (0.1 M phosphate buffer, pH 7.8; 0.1 % glucose, 0.1 % casamino acid and the same supplements as described above). The cell density was adjusted to 100 Klett photometer units at 540 nm and the culture was incubated at 28°C. When the culture attained the exponential growth phase, [³H] thymidine (10,400 mCi/m mole), [³H] uridine (6,200 mCi/m mole) and [¹⁴C] chlorella hydrolysate (24 mCi/m atom C) were added separately at a final concentration of 2 $\mu\text{Ci/ml}$ (for nucleic acid precursors) and 0.2 $\mu\text{Ci/ml}$ (for protein). One ml portions of culture removed at different time intervals were added to an equal volume of 20% chilled TCA (w/v). After 30 minutes, the precipitate was collected on a GF/C glass filter, washed with 25 ml of 10% TCA at 0°C, with 10 ml of ethyl alcohol containing 2% potassium acetate at 0°C, followed by final washing with diethyl ether at 0°C. The precipitate was dried and counted in a Liquid Scintillation Counter (Electronics Corporation of India Limited, India) using toluene based fluor. The definition of a unit of agrocin is the same as that which was used by IVANOVICS *et al.*¹⁰⁾ for megacin.

[A] Incorporation of [³H] thymidine:

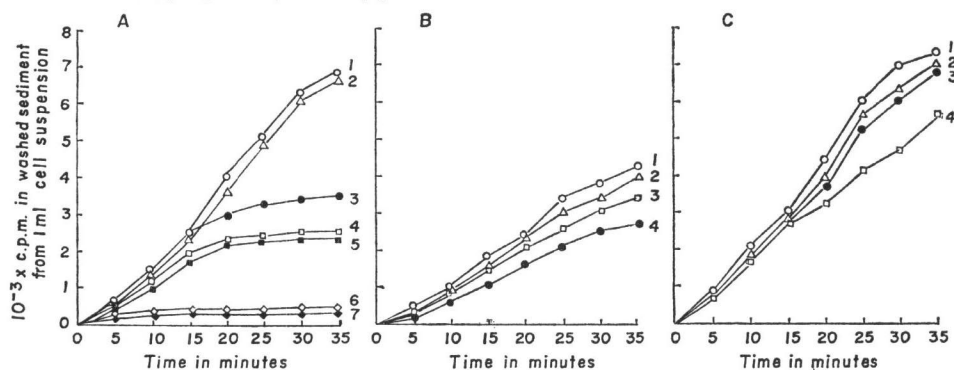
(1) Control; (2) Agrocin (50 units) added at 0 minutes; (3) Actinomycin D (10 $\mu\text{g/ml}$) added at 15 minutes; (4) Agrocin (50 units) preincubated for 15 minutes; (5) Trypsin (250 $\mu\text{g/ml}$) treated agrocin preincubated for 15 minutes; (6) Agrocin (50 units) preincubated for 30 minutes; (7) Actinomycin D (10 $\mu\text{g/ml}$) added at 0 minute.

[B] Incorporation of [³H] uridine:

(1) Control; (2) Agrocin (50 units) added at 0 minute; (3) Agrocin (50 units) preincubated for 30 minutes; (4) Agrocin (100 units) preincubated for 30 minutes.

[C] Incorporation of [¹⁴C] chlorella hydrolysate:

(1) Control; (2) Agrocin (50 units) added at 0 minute; (3) Agrocin (50 units) preincubated for 30 minutes; (4) Agrocin (100 units) preincubated for 30 minutes.



agrocin-resistant [*A. tumefaciens* S1005 (TIP Kerr 14) R, probably completely cured of plasmid] strains (obtained from Prof. J. SCHELL, Director, Laboratorium voor Genetica, Gent, Belgium) in relation to the synthesis of macromolecules like DNA, RNA and protein. Agrocin was prepared from *A. radiobacter* as described previously²⁾ and was further purified by passing through a DEAE-Sephadex column according to the method of ROBERTS *et al.*³⁾. Incorporation of radioactive precursors (³H] thymidine, [³H] uridine and [¹⁴C] chlorella hydrolysate for DNA, RNA and protein respectively) into acid-insoluble material was measured (Fig. 1). The results indicated that agrocin added at zero time could not affect the synthesis of any of the macromolecules, but when exponentially growing cultures of sensitive bacteria were preincubated with agrocin for 30 minutes, DNA synthesis was inhibited. In contrast, protein and RNA syntheses were completely unaffected at low concentrations and only partially affected at high concentrations. No effect on incorporation of any radioactive precursor was observed in the case of the resistant strain preincubated with agrocin (results not shown). This inhibition of DNA synthesis was not caused as a result of any DNase activity as no such DNase activity⁵⁾ could be detected in the agrocin preparation and this was further evident from Fig. 1, which shows that trypsin pretreatment of agrocin did not affect the activity of the bacteriocin preparation. Inhibition of DNA synthesis was accompanied by degradation of DNA in the case of both colicin E2⁶⁾ and megacin C⁷⁾. The action of agrocin was investigated in regard to its effect on the stability of DNA prelabelled with [³H] thymidine, but no degradation of preformed DNA of sensitive cells could be found. This was also confirmed by the estimation of cellular DNA in the presence of agrocin (Fig. 2). Agrocin was found to possess no effect on cell permeability as this was checked directly by determining the release of UV-absorbing material and the permeability of cells to the substrates of β -galactosidase in the presence of agrocin (data not given). β -Galactosidase was assayed according to the method of PARDEE *et al.*⁸⁾ using O-nitrophenyl- β -galactopyranoside as the substrate and toluene for breaking cells. The activity of β -galactosi-

Fig. 2. Effect of agrocin on the stability of cellular DNA

To label the DNA, sensitive cells were grown to the exponential phase in nutrient broth supplemented by [³H] thymidine. The cells were washed and resuspended in non-radioactive medium to the original cell density as described earlier. The cells were incubated for 30 minutes at 28°C to ensure that the radioactive nucleotides were incorporated into cellular DNA. Thereafter, the cell suspensions were distributed into flasks containing agrocin or saline (control). At suitable intervals, 1 ml samples were withdrawn and the radioactivity retained by the cells was measured as described above. The filtrate (0.1 ml) containing the acid-soluble material was dried on a disc of filter paper (Whatman no. 1) and the radioactivity was measured. DNA was also estimated according to BURTON's diphenylamine method¹¹⁾ modified by GILES and MYERS¹²⁾.

[A] [³H] thymidine in acid-precipitable fraction of cells:

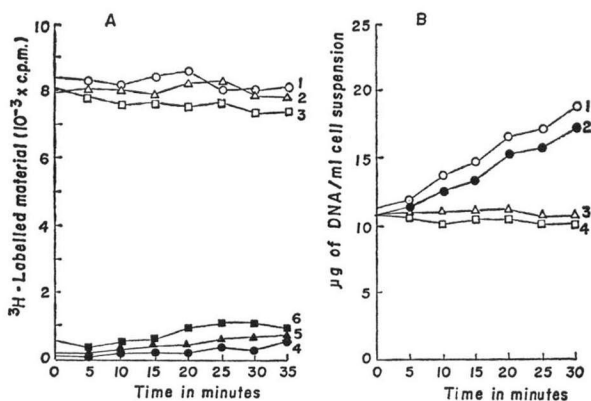
- (1) Control;
- (2) Agrocin (50 units) treated;
- (3) Preincubated with agrocin (50 units) for 30 minutes.

[³H] thymidine in acid-soluble fraction of cells:

- (4) Control;
- (5) Agrocin (50 units) treated;
- (6) Preincubated with agrocin (50 units) for 30 minutes.

[B] Estimation of cellular DNA:

- (1) Control cells;
- (2) Agrocin (50 units) treated cells;
- (3) Cells preincubated for 30 minutes with agrocin (50 units);
- (4) Agrocin (100 units).



dase in toluenized cells was used as a control. All these observations indicate that agrocin specifically affects DNA synthesis without causing DNA degradation and unlike many other bacteriocins it has no effect on cell permeability.

That agrocin requires a time of about 30 minutes in bringing about the observed inhibitory effect may be due to the fact that it is transformed or induced into some activated form during this period. The nature of this postulated transformation or induction processes is of course obscure, but for an understanding of the mode of action of agrocin this activation step seems to be one of the most important reactions to be clarified on the basis of future research.

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